Supplementary Materials and Methods

Proliferation assay

pLNCX-empty vector or pLNCX-N4ICD transduced cells were seeded into 96 well plates at a concentration of $5*10^3$ cells per well. Cell proliferation was measured by addition of 10µL of a 12mM MTT stock solution (Invitrogen Life Technologies) to 100 µL of RPMI media without phenol red (Gibco, Life Technologies). After 4 hours of incubation at 37°C, 100µL of a 0.01 M HCl 10% SDS solution were added an again incubated for 4 hours at 37°C. The assay was incubated at 37°C for 10 minutes and the absorbance was measured at 570 nm.

Adhesion assay

Cells were stained with 5µM Vybrant® DiI cell-labeling solution (Molecular Probes, Invitrogen) for 30 minutes at 37°C, washed with PBS and harvested using trypsin (Life Technologies). Cells were resuspended in DMEM medium containing 2% FCS, 2% L-Glutamine. Monolayers E-cadherin expressing HaCaT cells were incubated with 30.000 cells per well in 6-well-plates for 1 hour at 37°C. Non-adherent cells were removed by washing with PBS. Numbers of adherent cells were determined in triplicate wells using fluorescence microscopy.

Chromatin immunoprecipitation (ChIP) assay

ChIP was performed using SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling), following the manufacturer's protocol. Two different anti-CSL antibodies (D20 and H-50 see Supplementary Table 2) were used for chromatin precipitation while anti-Histone H3 and rabbit IgG antibody served as positive or negative control, respectively. All antibodies were diluted 1:50. Following the DNA purification isolated fragments were amplified using promoter specific primers (Supplementary Table 3) and visualized using agarose gel electrophoresis.

Supplementary Figure Legends

Supplementary Figure 1: Phenotypic changes induced by overexpression of N4ICD

A, Morphology of N4ICD transduced cells. Expression of N4ICD was confirmed by immunoblotting. β-Actin was used as a loading control. **B,** Migration of N4ICD transduced 451Lu. **C,** Invasion of N4ICD transduced 451Lu determined. **D,** Adhesion to keratinocytes of N4ICD transduced 451Lu determined by incubating cells with a monolayer of HaCaT. E, Proliferation of N4ICD transduced 451Lu, WM9 and WM164 determined by MTT assay. The error bars represent standard deviations from the mean. n.s. not significant, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$

Supplementary Figure 2: Correlation of Notch4 and EMT markers

A, Immunoblots of whole cell lysates of 451Lu transduced with pLNCX-empty vector or pLNCX-N4ICD constructs. β-Actin was used as loading control. **B,** Immunohistochemistry of primary melanomas (two upper panels) and a subcutaneous metastasis for E-cadherin (middle panel) and Notch4 (right panel). Left panel: unspecific secondary IgG antibody (x10; inserts x20).

Supplementary Figure 3: CSL directly binds Snail2 and Twist1 promoter regions

A, Immunoblots (left panel) or mRNA levels (right panel) of WM35 transfected with specific siRNA targeting CSL or scrambled control RNA (scRNA). **B,** Snail2 promoter region from -1500 bp to +500 bp and the Twist1 promoter region from -2000 bp to +500 bp relative to the transcriptional start site (TSS). Identified and randomly chosen potential CSL binding sites, the amplified ChIP fragments as well as the translational start (ATG) are indicated. **C,** EMSA of WM164 incubated with 20 nt fragments of Cy3 labeled wild type (wt-Cy3), Cy3 labeled wt together with 20 fold excess of unlabeled wt (wt-C3+20xwt unlabeled) or Cy3 labeled mutated (mut-Cy3) synthetic oligonucleotides and probed for CSL. **D,** ChIP assay was performed with WM9 and WM164 using antibodies targeting CSL (D20 and H-50), Histone

H3 and rabbit IgG for precipitation. Promoter specific primers amplified fragments of 229 bp (Snail2) and 316 bp (Twist1).

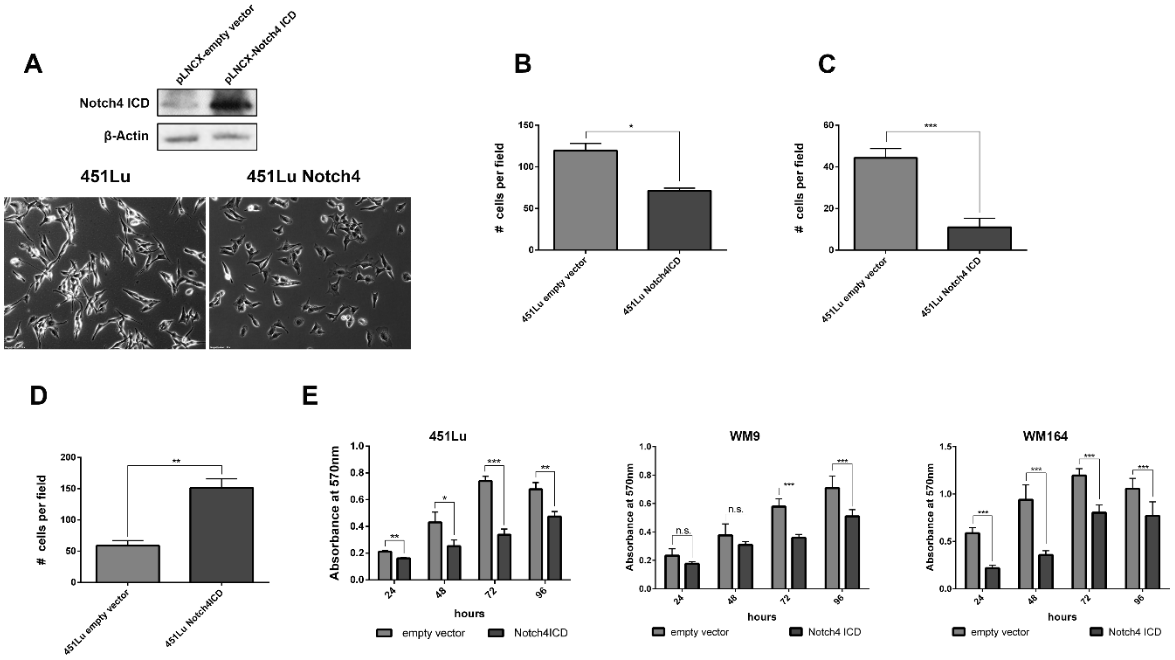
Supplementary Figure 4: Notch4 and Hey1/2 suppress Snail2 and Twist1 gene expression

A, Immunoblots (left panel) and mRNA (right panel) levels of Snail2 and Twist1 of WM9 and WM164 transfected with siRNA targeting Notch4 (siNotch4). **B,** Immunoblots (left panel) and mRNA (right panel) levels of Snail2 and Twist1 of WM35 transfected with siRNA targeting Notch4. **C,** Immunoblots of Hey1 and Hey2 of WM9 and WM164 transfected with siRNA targeting Notch4. **D,** Immunoblots (left panel) and mRNA (right panel) levels of Snail2 and Twist1 in WM35 transfected with siRNA targeting Hey1. **E,** Immunoblots (left panel) and mRNA (right panel) levels of Snail2 and Twist1 in WM35 transfected with siRNA targeting Hey2. The error bars represent standard deviations from the mean. Statistical significance of results was calculated using an unpaired t-test. n.s. not significant, * $P \le 0.05$, ** $P \le 0.01$, *** $P \le 0.001$

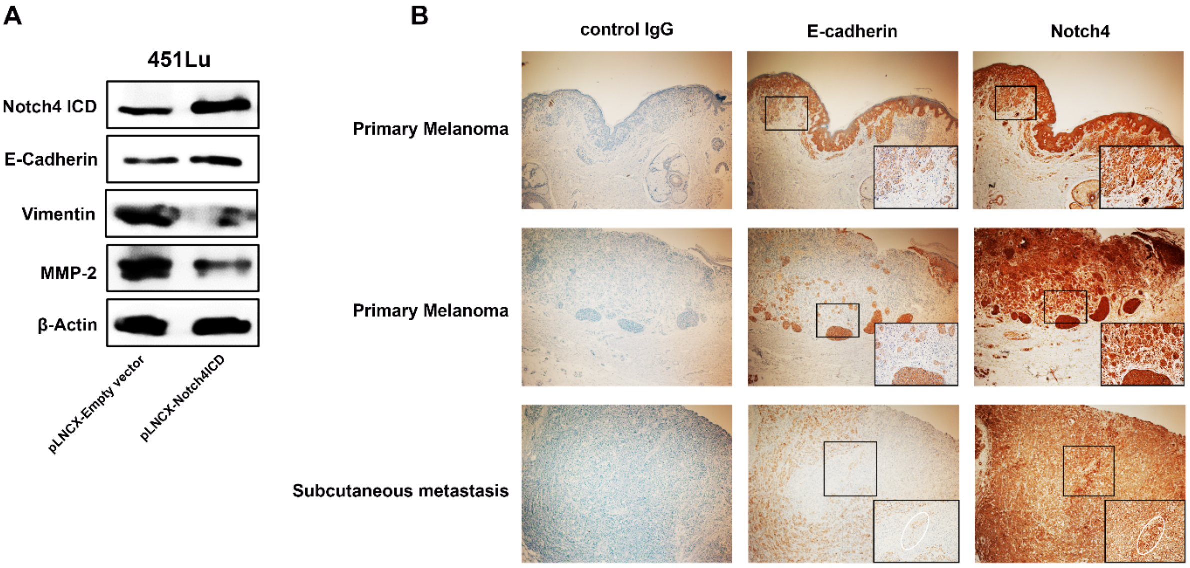
Supplementary Figure 5: Hey1/2 directly regulate Snail2 and Twist1 expression

A, Immunoblots of WM9 transduced with pLNCX-empty vector, pLNCX-N4ICD or pLNCX-N4ICD and siRNA targeting Hey1 (siHey1), Hey2 (siHey2) or scrambled control RNA (scRNA) **B,** Snail2 promoter region from -1500 bp to +500 bp and the Twist1 promoter region from -2000 bp to +500 bp relative to the transcriptional start site (TSS). Identified and randomly chosen potential E-Boxes and the translational start (ATG) are indicated.

Supplementary Figures



Supplementary Figure 1



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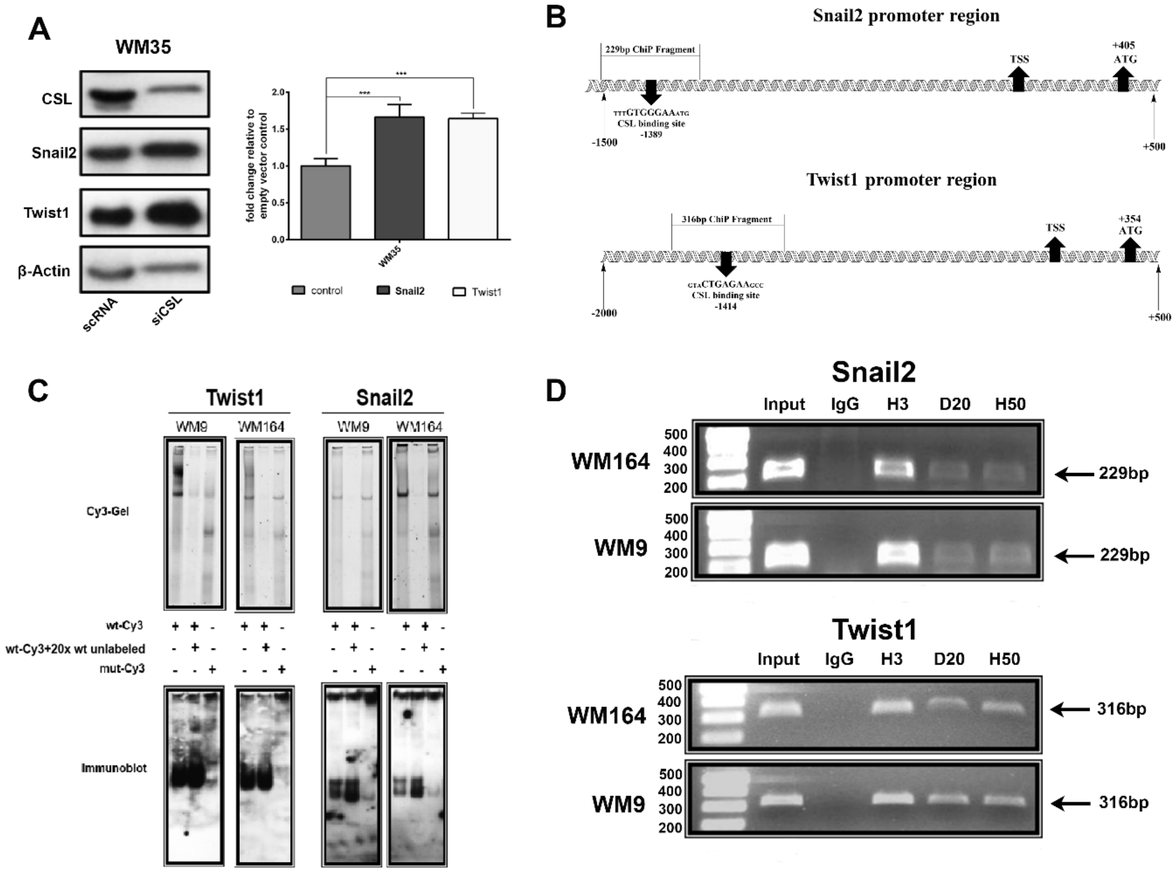
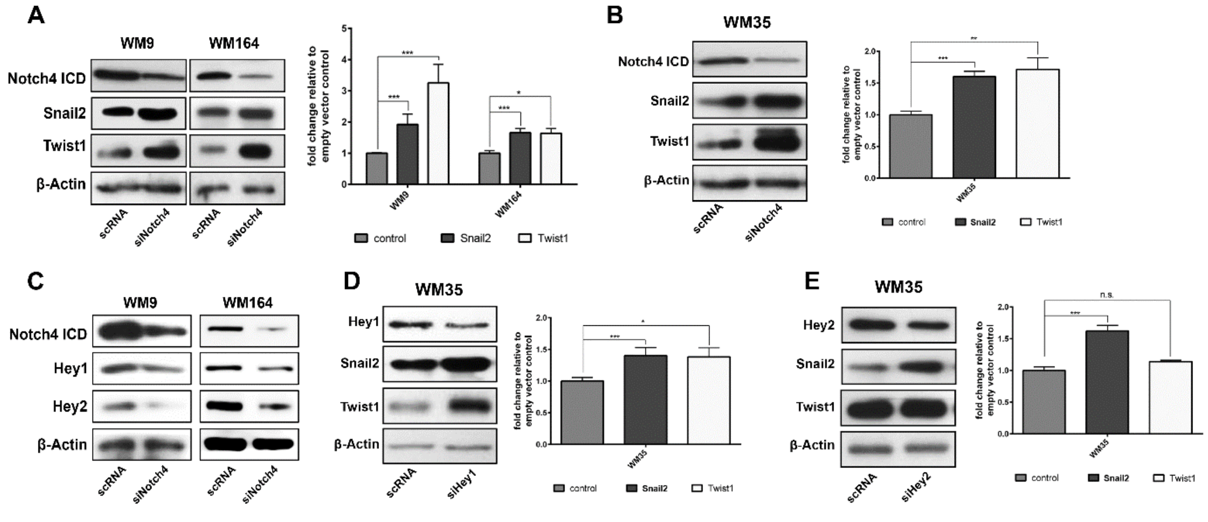
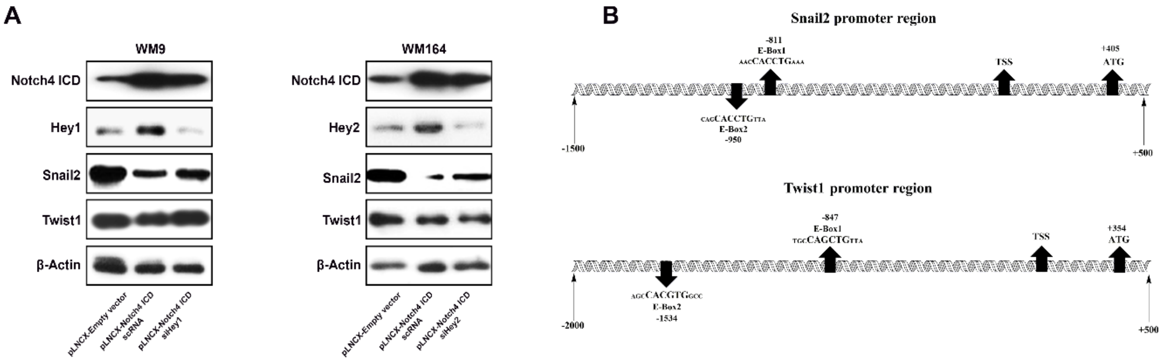


Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5

Supplementary Tables

Supplementary Table 1: Plasmids and siRNAs

Name	Vendor
SMARTpool: ON-TARGETplus HEY1 siRNA	Thermo Scientific/Dharmacon
SMARTpool: ON-TARGETplus HEY2 siRNA	Thermo Scientific/Dharmacon
SMARTpool: ON-TARGETplus NOTCH4 siRNA	Thermo Scientific/Dharmacon
SMARTpool: ON-TARGETplus RBPJ siRNA	Thermo Scientific/Dharmacon
Scrambled siRNA (scRNA)	QIAGEN
pCMV6-Hey1 (NM_012258 Human cDNA Clone	OriGene Technologies
pCMV6-Hey2 (NM_012259) Human cDNA Clone)	OriGene Technologies
pCMV6-XL5	OriGene Technologies
pLNCX-empty vector	Kindly provided by Dr. Aly Karsa (Departments of Experimental Medicine, Pathology and Laboratory Medicine, University of British Columbia and British Columbia Cancer Agency, Department of

	Medical Biophysics, British Columbia Cancer Agency, Vancouver, British Columbia V5Z 1L3, Canada)
pLNCX-N4ICD (Notch4ICD, amino acids 1476 to 2003, C-terminally HA-tagged)	Kindly provided by Dr. Aly Karsa (Departments of Experimental Medicine, Pathology and Laboratory Medicine, University of British Columbia and British Columbia Cancer Agency, Department of Medical Biophysics, British Columbia Cancer Agency, Vancouver, British Columbia V5Z 1L3, Canada
pVSV-G	Clontech
pGL3-basic	Promega
pSV-β-Galactosidase Control Vector	Promega
pGL3-PromSnail2 (sequence details see Supplementary Table 7)	Genscript
pGL3PromSnail2Mut (sequence details see Supplementary Table 7)	Genscript
pGL3-PromTwist1 (sequence details see Supplementary Table 7)	Genscript
pGL3PromTwist1Mut (sequence details see Supplementary Table 7)	Genscript

Supplementary Table 2: Antibodies

Primary antibody			**HRP conjugated secondary antibody**		
Target Protein	**Company**	**Dilution**	**Target**	**Company**	**Dilution**
Notch4	Cell Signaling	1:2000	Rabbit anti mouse	Dako	1:1000
Notch4	Abcam	1:150	Mouse and rabbit	Ventana	<50µg/ml
CSL	Gentex	1:1000	Goat anti rabbit	Santa Cruz	1:4000
CSL (D20)	Santa Cruz	1:50			
CSL (H50)	Santa Cruz	1:50			
E-cadherin	BD	1:500	Rabbit anti mouse	Dako	1:1000

	Bioscience				
E-cadherin	Dako	1:100	Mouse and rabbit	Ventana	<50µg/ml
Vimentin	Cell Signaling	1:1000	Goat anti rabbit	Santa Cruz	1:4000
MMP-2	Cell Signaling	1:1000	Goat anti rabbit	Santa Cruz	1:4000
Snail2	Santa Cruz	1:200	Donkey anti goat	Santa Cruz	1:4000
Twist1	Abcam	1:1000	Rabbit anti mouse	Dako	1:1000
β-Actin	Sigma	1:10000	Goat anti rabbit	Santa Cruz	1:4000
Hey1	Abcam	1:1000	Rabbit anti mouse	Dako	1:1000
Hey1	Abcam	1:1000	Goat anti rabbit	Santa Cruz	1:4000
Hey2	Abcam	1:1000	Goat anti rabbit	Santa Cruz	1:4000

Supplementary Table 3: Primers

Name	Vendor	Application
Snail2 (QT00044128)	QIAGEN	qPCR
Twist1 (QT00011956)	QIAGEN	qPCR
E-cadherin (QT00080143)	QIAGEN	qPCR
Vimentin (QT00095795)	QIAGEN	qPCR
Actin Fw: 5’CCACACTGTGCCCATCTACG3’ [1] Actin Rev: 5’AGGATCTTCATGAGGTAGTCAGTCAG3’ [1]	Ingenetix	qPCR
Hey1 Fw: 5’AGCCGAGATCCTGCAGATGA3’ [2] Hey1 Rev: 5’GCCGTATGCAGCATTTTCAG3’ [2]	Ingenetix	qPCR
Hey2 Fw: 5’AGATGCTTCAGGCAACAGGG3’ [2] Hey2 Rev: 5’CAAGAGCGTGTGCGTCAAAG3’ [2]	Ingenetix	qPCR
MMP2 Fw: 5’ATGCCGCCTTTAACTGGAG3’ [3] MMP2 Rev: 5’GGAAAGCCAGGATCCATTTT3’ [3]	Ingenetix	qPCR
Twist1 Fw 5’CCTGAGGTTTCCAAGACCAA3’ Twist1 Rev 5’CGTTTCAGGTCCATCCCTTA3’	Ingenetix	ChIP

Snail2 Fw 5’AGACTGTGTAGAGTGAAACAAGG3’ Snail2 Rev 5’TCTCCACACACAAACTGGAACCTG3’	Ingenetix	ChIP

Supplementary Table 4: EMSA sequences

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<tr><th colspan="2">Snail2 E-Box1</th></tr>
<tr><td>WT</td><td>Mut</td></tr>
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<tr><th colspan="2">Snail2 E-Box2</th></tr>
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<tr><td>WT</td><td>Mut</td></tr>
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<tr><td></td><td></td></tr>
<tr><th colspan="2">Snail2 CSL</th></tr>
<tr><td>WT</td><td>Mut</td></tr>
<tr><td>5´ TGTTTTGTGGGAAATGGAGT 3’</td><td>5´ TGTTTTGTGCTGCATGGAGT 3´</td></tr>
<tr><th colspan="2">Twist1 CSL</th></tr>
<tr><td>WT</td><td>Mut</td></tr>
<tr><td>5´ TCCGTACTGAGAAGCCCATG 3´</td><td>5´ TCCGTCAGACTCCTCCCATG 3’</td></tr>
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Supplementary Table 5: Sequences of Luciferase fragments

Twist1 Luciferase fragment
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Mutated Twist1 Luciferase fragment
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Snail2 Luciferase fragment

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CCTCCCGCCGGACCGTT

Mutated Snail2 luciferase Fragment

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CTCCCGCCGGACCGTT
```

Supplementary Table 6: Fold regulation of 50 EMT markers

	Epithelial Markers		Mesenchymal Markers	
	Fold regulation	Standard deviation	Fold regulation	Standard deviation
AHNAK			-2,39	1,77
BMP1			0,833	1,64
CALD1*			4,25	1,26
CAMK2N1			0,276	1,42
CAV2	-1,70	0,94		
CDH1*	26,04	24,08		
CDH2			0,444	1,81
COL1A2			-0,287	1,72
COL3A1			0,493	1,77
COL5A2			2,27	1,61
DSP*	284,49	100,34		
FGFBP1*	4,69	7,30		
FN1*			12,80	5,57
FOXC2			-2,22	1,19
GNG11			-1,49	0,36
GSC*			159,94	104,81
IGFBP4			2,39	1,22
IL1RN*	5,05	6,15		
ITGA5			-2,10	0,90
ITGAV			-1,11	2,15
KRT19	-3,38	3,47		
MMP2*			-7,73	4,86
MMP3			-2,34	1,69
MMP9*			6,82	4,55
MSN			-0,709	1,49
MST1R	8,61	20,10		
NUDT13	-1,85	0,25		
OCLN*	2,89	0,85		
DESI1	0,290	1,30		
RGS2*	-3,50	0,34		
SERPINE1			2,11	1,62
SNAI1*			-2,38	0,77

SNAI2*			-5,76	3,52
SNAI3			3,30	5,87
SOX10			-2,12	0,80
SPARC			-1,58	0,69
SPP1*	4,78	1,53		
STAT3			0,0381	1,77
TCF4			-0,578	1,86
TFPI2	0,472	1,29		
TIMP1			-2,15	1,10
TMEFF1			-0,587	1,71
TMEM132A			0,452	1,82
TSPAN13	-2,18	0,26		
TWIST1*			-2,69	0,53
VCAN			2,15	2,87
VIM			-2,08	0,79
VPS13A			0,735	1,59
WNT5A*			119,22	21,80
WNT5B*			-2,64	1,44

* indicates significantly changed expression. Fold regulation between -1 and 1 are considered as no discernible tendency.

Supplementary Table 7: Immunohistochemical expression of E-cadherin and Notch4 in melanocytic lesions.

Lesions (n)	**E-cadherin**				**Notch4**			
	-	+	++	+++	-	+	++	+++
Primary melanomas (30)	5 (17%)	3 (10%)	19 (63%)	3 (10%)	1 (3%)	1 (3%)	4 (14%)	24 (80%)
	Spearman´s rho = 0,367 (p≤0,05)							
Cutaneous subcutaneous metastases (30)	13 (43%)	6 (20%)	9 (30%)	2 (7%)			8 (27%)	22 (73%)
	Spearman´s rho = 0,366 (p≤0,05)							

Supplementary References

1. Rahman, K.W., et al., *Gene expression profiling revealed survivin as a target of 3,3'-diindolylmethane-induced cell growth inhibition and apoptosis in breast cancer cells.* Cancer Res, 2006. **66**(9): p. 4952-60.
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